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13. ABSTRACT (Maximum 200 Words) The breast cancer susceptibility gene BRCA1 is mutated in many cases of familial breast and ovarian cancer. BRCA1 plays a role in DNA repair, centrosomal function, ubiquitination, and the transactivation of genes. The phosphorylation status of BRCA1 changes throughout the cell cycle, and upon DNA damage. Phosphorylation may, therefore, play an important role in the regulation of BRCA1 function. We have performed a yeast two-hybrid study in order to identify proteins that interact with BRCA1 and have identified Protein Phosphatase 1 (PP1), a serine threonine phosphatase. PP1 and BRCA1 co-immunoprecipitate, and a GST pull down assay has identified the region within BRCA1 that is involved in the interaction. Colocalization studies suggest the proteins may be interacting in early M phase within the cell nucleus. Cell lines have been constructed that express the epitope tagged PP1 isoforms α , β or γ in order to identify, by immunofluorescence, which isoform is primarily associating with BRCA1. Mutational analysis of PP1, and identification of altered expression levels of PP1 in breast tumors, is also being performed. Characterization of the interaction of BRCA1 and PP1 could identify important ways in which BRCA1 function is controlled, and may suggest new roles for BRCA1 in the cell.			
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INTRODUCTION:

The breast cancer susceptibility gene BRCA1 is mutated in many cases of breast and ovarian cancer. The characterization of an interacting protein that may affect the function of BRCA1 will increase our understanding of the function of this gene. I have identified Protein Phosphatase 1 β , a serine/threonine phosphatase, as a BRCA1 interacting protein using a yeast two-hybrid screen. PP1 has 3 isoforms - α , β and γ - which are 97% conserved across the catalytic domain. Therefore, it is possible that any of the PP1 isoforms may be interacting with BRCA1.

The phosphorylation status of BRCA1 changes throughout the cell cycle, with a hypophosphorylated form being found from G1 to S phase of the cell cycle. Additionally, a hypophosphorylated form of BRCA1 has been identified at the centrosomes during M phase of the cell cycle (5). Mutant mouse cells containing BRCA1 lacking exon11 have been shown to have aneuploidy and problems with chromosome separation (7). In addition, PP1 α has recently been shown to interact with and dephosphorylate BRCA1 (6). The phosphorylation status of the protein may, therefore, have important consequences for its function. The identification and characterization of the interaction of BRCA1 and PP1 could help to identify how the BRCA1 protein functions within the cell. This project hopes to further characterize the interaction of BRCA1 and PP1, and to identify the potential significance of this interaction in the development of breast cancer.

BODY:

SPECIFIC AIM 1:

Identification of the Region of Interaction between BRCA1 and Protein Phosphatase 1 (PP1)

Task 1: In order to identify a region within BRCA1 that interacts with PP1, fragments of BRCA1 fused to GST epitope tags (Figure 1a) have been constructed. Equal amounts of GST-tagged protein produced in bacteria were added to an equal amount of 293 human embryonic kidney cell lysate. The GST-protein and any bound proteins were isolated using glutathione sepharose beads. After washing, GST-proteins and any interacting proteins were eluted from the beads and electrophoresed on an SDS-PAGE gel. A Western blot was performed on the gel using an antibody to protein phosphatase 1 (PP1 E-9, Santa Cruz). Results indicate that BRCA1 fragment 4 (BR-4) interacts with PP1 (Figure 1b).

BR-4 contains a putative PP1 interacting domain KVTF (1). Previous studies have shown that mutating the Valine or Phenylalanine residues to Alanine disrupts the interaction of PP1 with associated proteins (3). Creation of GST-fused BR-4 with either a V-A or F-A mutation shows decreased binding of PP1 to BR-4 (Figure 1c). Further examination of this is required to quantitate the degree of the effect of the mutations on the interaction, and to determine the effect of a double mutant, or removal of the entire KVTF domain, on the interaction of BRCA1 and PP1. Identification of the region of BRCA1 that is required to interact with PP1 is of benefit, as studies using BRCA1 that

lacks the interacting domain can be performed in cells without wild-type BRCA1 to identify any affect that the loss of the BRCA1-PP1 interaction may have.

In addition to the GST-pull down experiments, the association of BRCA1 and PP1 has been confirmed using 293 human embryonic kidney cells transfected with CMV-BRCA1 (to increase the expression of BRCA1) and Flag-epitope tagged PP1 α , β or γ . Immunoprecipitation using antibodies to either BRCA1 (Ab-1 and Ab-3, Oncogene) or the Flag epitope tag (M-2, Sigma) was done, immunoprecipitates were electrophoresed and a Western blot was performed. Blots were probed using antibodies to either BRCA1 or Flag. Results indicate that PP1 is able to co-immunoprecipitate BRCA1, and BRCA1 is also able to co-immunoprecipitate PP1 (Figure 2). The negative control (Flag-tagged Laf-4 (a nuclear protein not expected to interact with BRCA1)) showed no association with BRCA1. The strongest interaction was observed between PP1 α and BRCA1, as has previously been shown by Liu *et al* (2002). As PP1 α localizes to the centrosome during mitosis, dephosphorylation of BRCA1 by PP1 α at the centrosomes may be required for proper chromosome separation to occur.

Tasks 2 and 3: Development of vectors containing PP1 fused to a GST epitope tag, and determination of the region of PP1 that interacts with BRCA1, have not yet been completed, as PP1 is insoluble when produced in bacteria using the vectors created thus far. Therefore, additional vectors must be constructed in order to complete the identification of the region of PP1 that interacts with BRCA1.

SPECIFIC AIM 2:

Analysis of the cellular and temporal localization patterns of BRCA1 and PP1

Tasks 1 and 2: To identify where in the cell and when during the cell cycle the interaction between BRCA1 and PP1 is occurring, colocalization experiments have been performed using NIH-3T3 cells and antibodies to endogenous PP1 (FL-18, Santa Cruz) or BRCA1 (Ab-1, Oncogene). Fluorescent tagged secondary antibodies to either the mouse or rabbit primary antibodies were used to visualize the proteins. The 3T3 cells were synchronized using double thymidine selection, and the degree of synchronization was determined by FACS analysis. There appears to be colocalization of BRCA1 and PP1 in 2 to 3 large punctate nuclear dots (figure 3). Although perfect synchronization was not achieved, the appearance of the chromosomes suggests they may be in the process of condensing, indicating that the cells may be in early M phase. Although verification of these results is necessary, this suggests BRCA1 may be colocalizing with PP1 at the centrosomes in early M phase. Improved cell synchronization as well as colocalization of the BRCA1/PP1 complex with centrosomal markers such as γ -tubulin will clarify these results.

To further elucidate the role of the specific PP1 isoforms within the cell, SKOV3 ovarian cancer cells have been created that stably express Flag-epitope tagged PP1 α , β or γ . These studies should allow the identification of the specific PP1 isoform that interacts with BRCA1, and will help to clarify the roles that the individual PP1 isoforms have within the cell.

Task 3: Investigation of the effect of γ -irradiation, as well as other types of DNA damaging agents on the colocalization of BRCA1 and PP1 will be performed upon completion of the analysis of the SKOV3 PP1-expressing cell lines.

SPECIFIC AIM 3:

Mutational Analysis of BRCA1 and PP1

Tasks 1 and 2: To identify an effect of tumor-associated BRCA1 missense mutations on the interaction of BRCA1 and PP1, a yeast two-hybrid mating assay was performed. Yeast were transformed with BRCA1 missense mutants (Q356R, I 379M, E1038G, T826K) that were ligated into the pAS-1 DNA binding domain yeast two hybrid vector. These yeast then were mated with yeast that had been transformed with PP1 β ligated into a pACT2-Activating Domain vector to determine if the missense mutation in BRCA1 affected the interaction of BRCA1 with PP1. The strength of the BRCA1-PP1 interaction was determined by analyzing β -galactosidase levels, as the lacZ reporter gene is activated upon association of the two proteins. None of the tumor-associated missense mutations that were tested had an affect on the interaction of BRCA1 and PP1. Analysis of other missense mutations (**tasks 3 and 4**) within the BR-4 region (see figure 1a) must be performed to fully determine if tumor associated BRCA1 missense mutations have an affect on the interaction of the two proteins

Task 5: Single Strand Conformation Polymorphism analysis (SSCP) was performed on exons 5, 6 and 7 of PP1 β . These are the exons that are primarily involved in binding of PP1 to other interacting proteins (2, 3, 4). 55 breast tumors were analyzed, and no polymorphisms were found. Therefore, **Tasks 6 and 7** (identification of the affect of PP1 mutations on the interaction of PP1 and BRCA1) have not been completed. However, as the strongest interaction appears to be between BRCA1 and PP1 α , as seen by coimmunoprecipitation experiments, this isoform will also be analyzed using SSCP.

Task 8: Primers are being optimized for PP1 α , β and γ in order to perform quantitative RT-PCR. Once optimized, breast tumor RNA will be analyzed to identify any changes in PP1 expression levels.

SPECIFIC AIM 4:

Study of the association of BRCA1 and PP1 with other BRCA1 associated proteins.

Tasks 1 and 2: No work has yet been performed on Specific Aim 4 to identify if any other known BRCA1 interacting proteins also associate with the BRCA1-PP1 complex. Once colocalization experiments have been completed on the BRCA1 and the PP1 isoforms, this specific aim will be completed.

KEY RESEARCH ACCOMPLISHMENTS:

- Confirmed the interaction of BRCA1 and PP1 with reciprocal co-immunoprecipitation experiment, which indicated the strongest interaction is between BRCA1 and PP1 α .
- Identified a region within BRCA1 that interacts with PP1 and identified a potential PP1 interaction motif (KVTF) within that region
- Preliminary results indicate colocalization of BRCA1 and PP1 within punctate nuclear dots, possibly during early M phase of the cell cycle
- No effect on the interaction of BRCA1 and PP1 was identified when 5 BRCA1 tumor associated mutations were tested.
- No mutations within PP1 β were found within the region tested by SSCP. However, the other PP1 isoforms will be tested, particularly PP1 α as it strongly associates with BRCA1.

REPORTABLE OUTCOMES:

Abstracts presented:

Hendry, S.L., Andrulis, I.L. "Analysis of the interaction of BRCA1 with Protein Phosphatase 1 β ". Proc.Amer. Assoc.Cancer Res. 43:4900, 2002.

Hendry, S.L., Andrulis, I.L. "Investigation of the Interaction of BRCA1 and Protein Phosphatase 1" Proc.Amer.Assoc.Cancer Res. (1st Ed.) 44:4949, 2003.

Cell lines created: SKOV3 ovarian cancer cell lines expressing either the PP1 α , β or γ isoforms fused to a Flag epitope tag, or the Flag epitope tag vector alone (to be used as a negative control).

CONCLUSIONS:

Phosphorylation controls the function of a large number of proteins, many of which are involved in cell cycle regulation or DNA repair, and are implicated in cancer. Characterization of the interaction of BRCA1 and a serine/threonine phosphatase PP1, which may have an affect of the function of BRCA1 itself or on BRCA1-associated proteins, will greatly increase our understanding of the regulation of BRCA1. Co-immunoprecipitation studies have identified that the interaction with BRCA1 appears to be strongest with the isoform PP1 α . Additionally, the region within BRCA1 where the

interaction is occurring has been identified, and it contains a putative PP1 interaction motif, KVTF. When the PP1 interaction motif is mutated, the interaction of BRCA1 and PP1 appears to decrease. PP1 and BRCA1 colocalize within the nucleus of the cell, possibly during early M phase. Further characterization of the colocalization of the proteins, as well as analysis of the effect of DNA damaging agents and colocalization of PP1 and BRCA1 with other known BRCA1-associating proteins is required. This will be facilitated by the creation of SKOV3 cell lines expressing one of the 3 PP1 isoforms. Mutational and expression studies of PP1 have not yet identified any alterations in gene sequence or expression, however these results are preliminary.

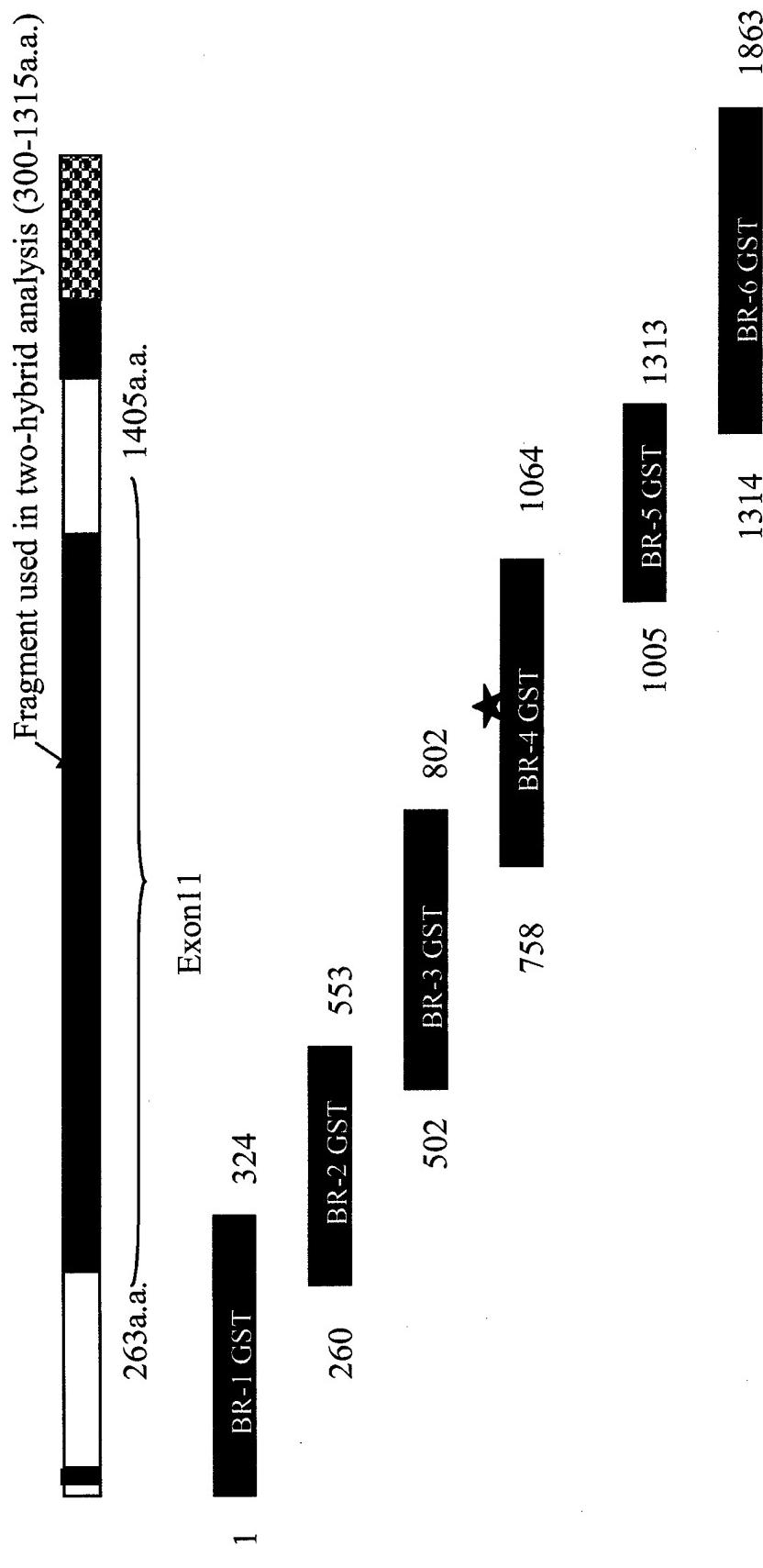
Characterization of this interaction increases our understanding of the roles for BRCA1 in the cell, and how phosphorylation may control the function of the protein. Insight into the regulation of BRCA1 will greatly improve our understanding of the role of BRCA1 in breast cancer.

REFERENCES

1. Damer, C.K., J. Partridge, W.R. Pearson, and T.A. Haystead. 1998. Rapid identification of protein phosphatase 1-binding proteins by mixed peptide sequencing and data base searching. Characterization of a novel holoenzymic form of protein phosphatase 1. *J.Biol.Chem.* **273**:24396-24405.
2. Egloff, M.P., P.T.W. Cohen, P. Reinemer, and D. Barford. 1995. Crystal structure of the catalytic subunit of human Protein Phosphatase 1 and its complex with tungstate. *J.Mol.Biol.* **254**:942-959.
3. Egloff, M.P., D.F. Johnson, G. Moorhead, P.T. Cohen, P. Cohen, and D. Barford. 1997. Structural basis for the recognition of regulatory subunits by the catalytic subunit of protein phosphatase 1. *EMBO J.* **16**:1876-1887.
4. Goldberg, J., H-b. Huang, Y-g. Kwon, P. Greengard, A.C. Nairn and J. Kuriyan. 1995. Three-dimensional structure of the catalytic subunit of protein serine/threonine phosphatase-1. *Nature.* **376**:745-53.
5. Hsu, L.C. and R.L. White. 1998. BRCA1 is associated with the centrosome during mitosis. *ProcNatlAcadSciUSA.* **95**:12983-8.
6. Liu, Y., D.M. Virshup, R.L. White, and L.C. Hsu. 2002. Regulation of BRCA1 phosphorylation by interaction with protein phosphatase 1 alpha. *Cancer Research* **62**:6357-61.
7. Xu, X., Z. Weaver, S.P. Linke, C. Li, J. Gotay, X.W. Wang, C.C. Harris, T. Ried and C.X. Deng. 1999. Centrosome amplification and a defective G2-M cell cycle checkpoint induce genetic instability in BRCA1 exon 11 isoform-deficient cells. *Mol.Cell* **3**:389-95.

Appendix

Figure 1a GST-Expression Vectors Constructed to Identify the Region of Interaction



6 vectors were constructed with fragments of BRCA1 fused in frame to the GST epitope (vector). These plasmids were transformed into bacterial cells to express the fusion proteins. The ★ represents the putative PP1 interacting domain KVTF (898-901.)

GST pull down to identify the region in BRCA1 interacting with PP1

Figure 1b

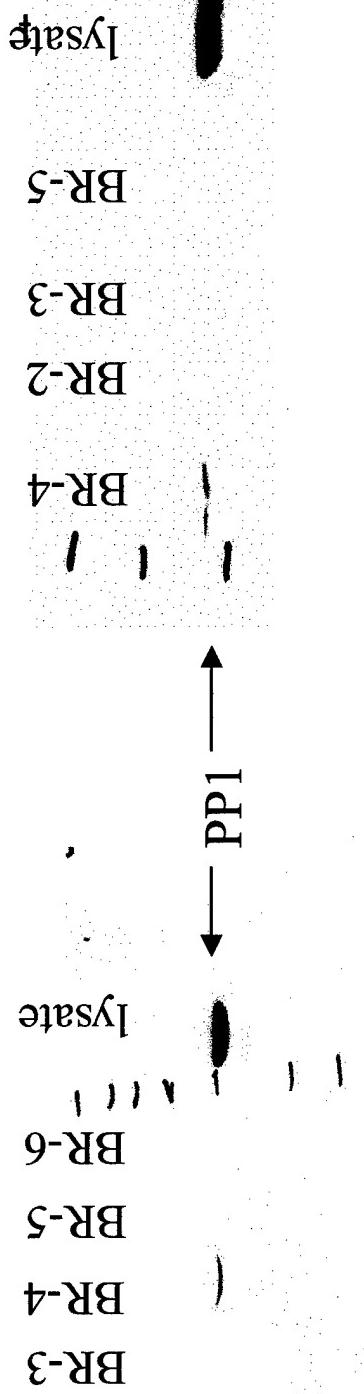


Figure 1c

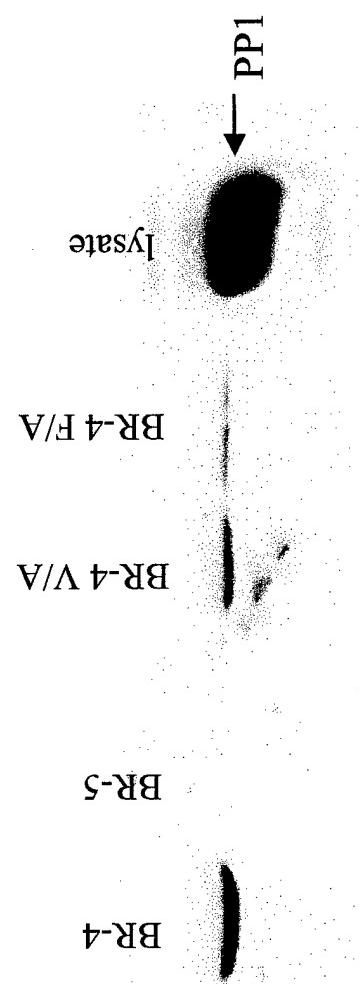
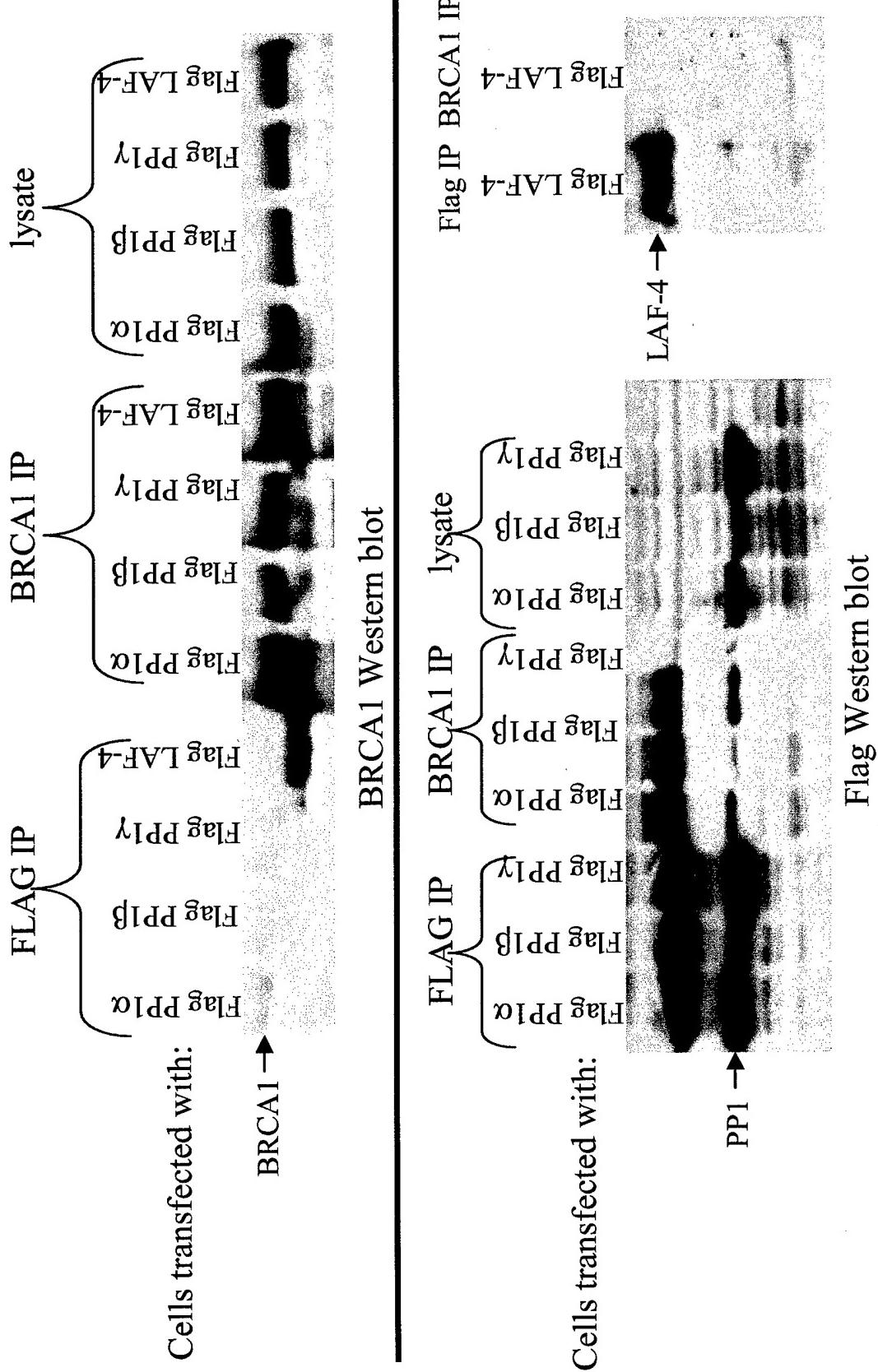


Figure 1b: An equal amount of cell lysate was added to 0.5ug of GST-bound proteins, and was incubated overnight. Samples were washed and electrophoresed on a 12% SDS-PAGE gel, transferred, and probed with an antibody to PP1 (E-9, Santa Cruz). BR-4 interacts with PP1. **Figure 1c:** Mutations in the putative interaction domain (V899A, F901A) decrease the interaction of BRCA1 and PP1.

Figure 2

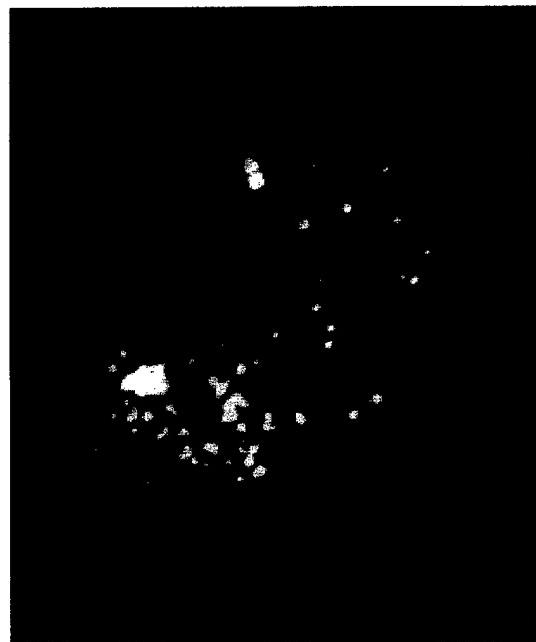
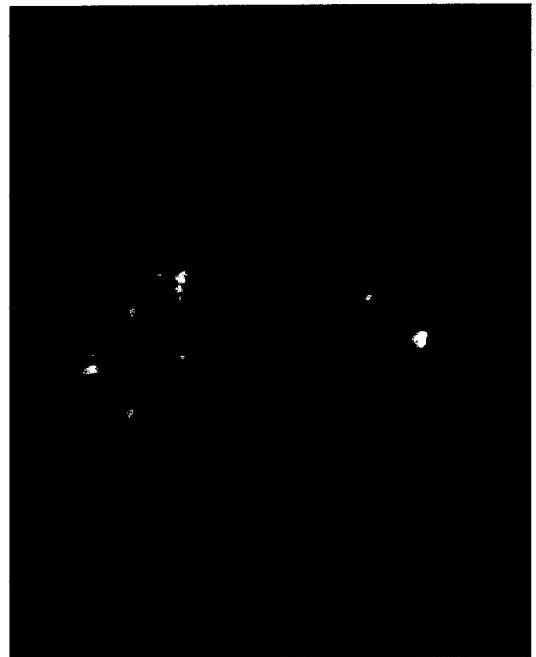
Co-immunoprecipitation of BRCA1 and PP1



Coimmunoprecipitation of 293 human embryonic kidney cells with BRCA1 (Ab-1, Ab-3 (Oncogene)), or Flag (M-2, (Sigma)). Cells are transfected with CMV-BRCA1 and either Flag-PP1 α , β , γ or Flag-Laf4 (negative control). Immunoprecipitation of lysates with BRCA1 shows co-immunoprecipitation of PP1 α , β and γ but not Laf-4. Conversely immunoprecipitation of Flag epitope tagged proteins indicates co-immunoprecipitation of BRCA1 by PP1 α , β and γ but not Laf-4. PP1 α appears to coimmunoprecipitate BRCA1 more strongly than the other PP1 isoforms.

Figure 3

Colocalization of BRCA1 and PP1



Two different 3T3 cells stained for BRCA1 (green) and PP1 (red) indicate nuclear colocalization in punctate dots, possibly during early M phase of the cell cycle. 9 hours after release from synchronization, 3T3 cells were fixed with 3% paraformaldehyde, permeabilized with 0.2% triton and stained with BRCA1 monoclonal Ab (Ab-1, Oncogene), or rabbit polyclonal PP1Ab (FL-18, Santa Cruz). Hoescht staining (blue) indicates the nucleus.